(2006)). Although non-integrating viral vectors, such as integrase defective lentiviral vectors or adeno-associated viral (AAV) vectors, have been used to deliver large donor nucleic acid sequences to cells, these vectors require viral infection and cause off-target effects. Therefore, compositions and methods for targeted insertion of large nucleotide sequences into the genome of a cell are needed.

BRIEF SUMMARY

[0004] The present disclosure is directed to compositions and methods for editing the genome of a cell. The inventors have discovered that large nucleotide sequences, for example, sequences greater than about 200 nucleotides in length, can be inserted into a targeted region in the genome of a cell. In some methods, integration of sequences greater than about 200 nucleotides in length occurs while reducing off-target effects and/or reducing loss of cell viability.

[0004a] In some aspects, the present disclosure provides an *in vitro* method of editing the genome of a primary human immune cell, the method comprising: a) providing a Cas9 ribonucleoprotein complex (RNP)-DNA template complex comprising: (i) the RNP, wherein the RNP comprises a Cas9 nuclease domain and a guide RNA, wherein the guide RNA specifically hybridizes to a target region of the genome of the cell, and wherein the Cas9 nuclease domain cleaves the target region to create an insertion site in the genome of the cell; and (ii) a double-stranded or single-stranded DNA template, wherein the size of the DNA template is greater than about 200 nucleotides or base pairs in length, wherein the 5' and 3' ends of the DNA template comprise nucleotide sequences that are homologous to genomic sequences flanking the insertion site,and wherein the molar ratio of RNP to DNA template in the complex is from about 3:1 to about 100:1; and b) introducing the RNP-DNA template complex into the cell.

[0004b] In some aspects, the present disclosure provides an isolated population of primary human immune cells, comprising a plurality of immune cells that comprise at least one heterologous DNA template non-virally inserted into a target region of the genome of the cell, wherein the size of the at least one heterologous DNA template is greater than 200 nucleotides or base pairs in length.

[0004c] In some aspects, the present disclosure provides an isolated population of primary human immune cells, comprising a plurality of immune cells that comprise at least one

heterologous DNA template in a target region of the genome of the cell, wherein the size of the at least one heterologous DNA template is greater than 200 nucleotides or base pairs in length, and wherein the primary human immune cells do not comprise a viral vector for introducing the at least one heterologous DNA template into the primary human immune cells.

[0004d] In some aspects, the present disclosure provides a primary human immune cell produced by the method of the disclosure.

[0004e] In some aspects, the present disclosure provides a use of the population or cell according to the disclosure in the manufacture of a medicament for treating a cancer, an infectious disease, or autoimmune disease in a subject.

[0005] In some examples, the present disclosure provides a method of editing the genome of a cell, the method comprising: a) providing a Cas9 ribonucleoprotein complex (RNP)-DNA template complex comprising: (i) the RNP, wherein the RNP comprises a Cas9 nuclease domain and a guide RNA, wherein the guide RNA specifically hybridizes to a target region of the genome of the cell, and wherein the Cas9 nuclease domain cleaves the target region to create an insertion site in the genome of the cell; and (ii) a double-stranded or single-stranded DNA template, wherein the size of the DNA template is greater than about 200 nucleotides, wherein the 5' and 3' ends of the DNA template comprise nucleotide sequences that are homologous to genomic sequences flanking the insertion site,and wherein the molar ratio of RNP to DNA template in the complex is from about 3:1 to about 100:1; and b) introducing the RNP-DNA template complex into the cell.

[0006] In some examples, the DNA template is a linear DNA template. In some examples, the DNA template is a single-stranded DNA template. In certain examples, the single-stranded DNA template is a pure single-stranded DNA template.

[0007] In some examples, the RNP-DNA template complex is formed by incubating the RNP with the DNA template for about one to about thirty minutes, at a temperature of about 20^{o} to 25^{o} C. In some examples, the RNP-DNA template complex and the cell are mixed prior to introducing the RNP-DNA template complex into the cell.

[0008] In some examples, the RNP comprises a Cas9 nuclease. In some examples, the RNP comprises a Cas9 nickase. In some examples, the RNP-DNA template complex comprises at least two structurally different RNP complexes. In some examples, the at least two structurally different RNP complexes contain structurally different Cas9 nuclease domains In some examples, the at least two structurally different RNP complexes contain structurally different guide RNAs. In some examples, wherein the at least two structurally different RNP complexes contain structurally different guide RNAs, each of the structurally different RNP complexes comprises a Cas9 nickase, and the structurally different guide RNAs hybridize to opposite strands of the target region.

[0009] In some examples, introducing the RNP-DNA template complex into the cell comprises electroporation. In some examples, the molar ratio of of RNP to DNA template is from about 5:1 to about 15:1. In some examples, the molar ratio of RNP to DNA template is from about 5:1 to about 10:1. In some examples, the molar ratio of RNP to DNA template is from about 8:1 to about 12:1. In some examples, the DNA template is at a concentration of about 2.5 pM to about 25 pM. In some examples, the size of the DNA template is greater than about 1kb. In some examples, the amount of DNA template is about 1 µg to about 10µg.

[0010] In some examples, the RNP-DNA template complex is introduced into about 1 x 10^5 to about 2 x 10^6 cells. In some examples, the cell is a primary hematopoietic cell or a primary hematopoietic stem cell. In some examples, the primary hematopoietic cell is an immune cell. In some examples, the immune cell is a T cell. In some examples, the T cell is a regulatory T cell, an effector T cell, or a naïve T cell. In some examples, the T cell is a $CD8^+$ T cell. In some examples, the T cell is a $CD4^+CD8^+$ T cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] The present application includes the following figures. The figures are intended to illustrate certain embodiments and/or features of the compositions and methods, and to supplement any description(s) of the compositions and methods. The figures do not limit the scope of the compositions and methods, unless the written description expressly indicates that such is the case.

[0012] Figure 1 shows low cell viability after electroporation of high concentrations of naked DNA necessary to achieve a workable editing efficiency in a cell.

What is claimed is:

1. An *in vitro* method of editing the genome of a primary human immune cell, the method comprising:

   a) providing a Cas9 ribonucleoprotein complex (RNP)-DNA template complex comprising:

      (i) the RNP, wherein the RNP comprises a Cas9 nuclease domain and a guide RNA, wherein the guide RNA specifically hybridizes to a target region of the genome of the cell, and wherein the Cas9 nuclease domain cleaves the target region to create an insertion site in the genome of the cell; and

      (ii) a double-stranded or single-stranded DNA template, wherein the size of the DNA template is greater than about 200 nucleotides or base pairs in length, wherein the 5' and 3' ends of the DNA template comprise nucleotide sequences that are homologous to genomic sequences flanking the insertion site,and wherein the molar ratio of RNP to DNA template in the complex is from about 3:1 to about 100:1; and

   b) introducing the RNP-DNA template complex into the cell.

2. The method of claim 1, wherein the RNP-DNA template complex is formed by incubating the RNP with the DNA template for less than about one minute to about thirty minutes, at a temperature of about 20° to 25° C.

3. The method of claim 1 or 2, wherein the DNA template is a linear DNA template.

4. The method of claim 1 or 2, wherein the DNA template is a plasmid.

5. The method of any one of claims 1-4, wherein the DNA template is a single-stranded DNA template

6. The method of any one of claims 1-4, wherein the DNA template is a pure single-stranded DNA template.

7. The method of any one of claims 1-3, wherein the DNA template is a double-stranded DNA template.

8. The method of any one of claims 1-7, wherein the DNA template comprises a gene.

9. The method of claim 8, wherein the DNA template encodes a chimeric antigen receptor (CAR).
10. The method of any one of claims 1-9, wherein the RNP-DNA template complex and the cell are mixed prior to introducing the RNP-DNA template complex into the cell.
11. The method of any one of claims 1-10, wherein the RNP comprises a Cas9 nuclease.
12. The method of any one of claims 1-10, wherein the RNP comprises a Cas9 nickase.
13. The method of any of claims 1-12, wherein the RNP-DNA template complex comprises at least two structurally different RNP complexes.
14. The method of claim 13, wherein the at least two structurally different RNP complexes contain structurally different guide RNAs.
15. The method of claim 14, wherein each of the structurally different RNP complexes comprises a Cas9 nickase, and wherein the structurally different guide RNAs hybridize to opposite strands of the target region.
16. The method of claim 13, wherein the at least two structurally different RNP complexes contain structurally different Cas9 nuclease domains.
17. The method of any one of claims 1-16, wherein the introducing comprises electroporation.
18. The method of any one of claims 1-17, wherein the molar ratio of RNP to DNA template is from about 5:1 to about 15:1.
19. The method of any one of claims 1-18, wherein the molar ratio of RNP to DNA template is from about 5:1 to about 10:1.
20. The method of any one of claims 1-19, wherein the molar ratio of RNP to DNA template is from about 8:1 to about 12:1.
21. The method of any one of claims 1-20, wherein the size of the DNA template is greater than about 300 nucleotides or base pairs in length.
22. The method of any one of claims 1-20, wherein the size of the DNA template is greater than about 1 kb in length.
23. The method of claim 22, wherein the size of the DNA template is greater than about 1.5 kb in length.

24. The method of any one of claims 1-23, wherein the DNA template is at a concentration of about 2.5 pM to about 25 pM.
25. The method of any one of claims 1-24, wherein the amount of DNA template is about 1 µg to about 10µg.
26. The method of any one of claims 1-25, wherein the cell is a primary hematopoietic cell or a primary hematopoietic stem cell,
27. The method of any one of claims 1-26, wherein the RNP-DNA template complex is introduced into about 1 x 10^5 to about 2 x 10^6 cells.
28. The method of any one of claims 1-27, wherein the immune cell is a T cell.
29. The method of claim 28, wherein the T cell is a regulatory T cell, an effector T cell, or a naïve T cell.
30. The method of claim 29, wherein the regulatory T cell, effector T cell, or naïve T cell is a $CD4^+$ T cell.
31. The method of claim 28, wherein the T cell is a $CD8^+$ T cell.
32. The method of claim 28, wherein the T cell is a $CD4^+CD8^+$ T cell.
33. An isolated population of primary human immune cells, comprising a plurality of immune cells that comprise at least one heterologous DNA template non-virally inserted into a target region of the genome of the cell, wherein the size of the at least one heterologous DNA template is greater than 200 nucleotides or base pairs in length.
34. An isolated population of primary human immune cells, comprising a plurality of immune cells that comprise at least one heterologous DNA template in a target region of the genome of the cell, wherein the size of the at least one heterologous DNA template is greater than 200 nucleotides or base pairs in length, and wherein the primary human immune cells do not comprise a viral vector for introducing the at least one heterologous DNA template into the primary human immune cells.
35. The population of claim 33 or 34, wherein the size of the DNA template is greater than about 300 nucleotides or base pairs in length.
36. The population of claim 33 or 34, wherein the size of the DNA template is greater than about 1 kb in length.
37. The population of claim 33 or 34, wherein the size of the DNA template is greater than about 1.5 kb in length.

38. The population of any one of claims 33-37, wherein the DNA template comprises a gene.
39. The population of claim 38, wherein the DNA template encodes a chimeric antigen receptor (CAR).
40. The population of any one of claims 33-39, wherein the immune cells are T cells.
41. The population of claim 40, wherein the T cells comprise regulatory T cells, effector T cells, or naïve T cells.
42. The population of claim 41, wherein the regulatory T cells, effector T cells, or naïve T cells are $CD4^+$ T cells.
43. The population of claim 40, wherein the T cells are $CD8^+$ T cells.
44. The population of claim 40, wherein the T cells are $CD4^+CD8^+$ T cells.
45. The population of any one of claims 33-44, wherein the primary immune cells do not comprise a viral vector.
46. The population of any one of claims 33-45, wherein at least 10% of the primary human immune cells comprise the DNA template in the target region.
47. The population of claim 46, wherein at least 20% of the primary human immune cells comprise the DNA template in the target region.
48. A primary human immune cell produced by the method of any one of claims 1-32.
49. The population or cell according to any one of claims 33-48 for use in therapy for a human subject.
50. The population or cell according to any one of claims 33-48, wherein the primary human immune cell or cells is/are to be obtained from the human subject.
51. Use of the population or cell according to any one of claims 33-48 in the manufacture of a medicament for treating a cancer, an infectious disease, or autoimmune disease in a subject.